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Anti-HBs antibody of normal human subjects predominantly binds 54 K and 60 K dalton HBs polypeptides.

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Abstract

The structure of hepatitis B virus surface antigen (HBs) recognized by anti-HBs antibody was analyzed by western blotting using anti-HBs sera obtained from normal subjects, from rabbits immunized with purified HBs and commercially available goat serum. The HBs used had 7 components of 24 K, 27 K, 33 K, 36 K, 39 K, 43 K and 67-72 K daltons. Goat anti-HBs serum bound all of these components, while human and rabbit anti-HBs sera bound only two components (60 K and 54 K daltons), which were hardly visible in the gel even by silver staining. Mixing the 24 K and 27 K components, and the 24 K and 43 K components without reducing reagent produced several polymerized forms of HBs components including 60 K and 54 K polypeptides, which were recognized by anti-HBs rabbit serum. Other combinations of HBs components did not yield any new polymeric forms. Thus, it was concluded that the formation of anti-HBs antibody in normal subjects might predominantly require an antigenic structure of polymeric forms of specific combinations of HBs polypeptides, other than previously known antigenic determinants.

KEYWORDS: HBs polypeptides, anti-HBs antibody, antigenic structure

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Anti-HBs Antibody of Normal Human Subjects Predominantly Binds 54 K and 60 K Dalton HBs Polypeptides

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The structure of hepatitis B virus surface antigen (HBs) recognized by anti-HBs antibody was analyzed by western blotting using anti-HBs sera obtained from normal subjects, from rabbits immunized with purified HBs and commercially available goat serum. The HBs used had 7 components of 24 K, 27 K, 33 K, 36 K, 39 K, 43 K and 67-72 K daltons. Goat anti-HBs serum bound all of these components, while human and rabbit anti-HBs sera bound only two components (60 K and 54 K daltons), which were hardly visible in the gel even by silver staining. Mixing the 24 K and 27 K components, and the 24 K and 43 K components without reducing reagent produced several polymerized forms of HBs components including 60 K and 54 K polypeptides, which were recognized by anti-HBs rabbit serum. Other combinations of HBs components did not yield any new polymeric forms. Thus, it was concluded that the formation of anti-HBs antibody in normal subjects might predominantly require an antigenic structure of polymeric forms of specific combinations of HBs polypeptides, other than previously known antigenic determinants.

Key words : HBs polypeptides, anti-HBs antibody, antigenic structure

Hepatitis B (HB) virus infection leads to the production of anti-HB surface antigen (anti-HBs) antibody as the neutralizing antibody late in the immune response in man. Passive immunization with anti-HBs antibody and vaccination with HBs are reported to provide equal protection against HBV infection (1-7). Antigenic structures of HBs recognized by anti-HBs human antibody was not fully understood. Anti-HBs serum generally contains the antibody against the group specific antigenic determinant *a* of HBs (4, 8) which was supposed to be one of the antigenic structure predominantly recognized by anti-HBs. Recent extensive analysis of HBs polypeptides, in agreement with the results of molecular biological investigation, has revealed

that HBs is constituted of at least 6 principal polypeptides with molecular weights of 24 K, 27 K, 33 K, 36 K, 39 K and 43 K daltons (9, 10). Some other larger polypeptides of 68 K, 72 K and 97 K are also suspected to be constituents of HBs (11, 12), but they have not been confirmed molecular biologically yet. The 6 principal polypeptides are known to carry the both group specific and subtype specific antigen epitopes, and be able to induce anti-HBs antibody production in experimental animals, though the antigenicity of the antibody induced by these polypeptides was weak (13-15). On the other hand, three-dimensional polymeric structure of HBs might be more antigenic than the individual components of HBs, since the fragment of HBs containing

the known principal polypeptides was reported to be more potent than the polypeptides themselves in producing anti-HBs antibody (16-18). The pre-S region also has strong antigenicity (19, 20). The fragment of HBs carrying strong antigenicity was considered to be a good material to investigate the structural properties of HBs antigen. As reported by others (16), however, it is difficult to prepare its enough amount for analytical purpose. In this study, we investigated the structural properties of HBs recognized by anti-HBs antibody generated in normal human subjects and the antigenicity of HBs polypeptides in polymeric form using reconstituted HBs polypeptides.

Materials and Methods

HBs antigen. Purified HBs prepared by the procedure of Gerin *et al.* (21) from HBs-positive healthy carriers was kindly donated by Dr. Nakamura, Fujirebio Inc. The purification procedure included removal of heavy HB virus (HBV) particles by ultracentrifugation, precipitation of HBs with 1.8 M ammonium sulfate and ion exchange chromatography. Screening and titration of HBs antigen were performed with the SERODIA-HBs antigen kit (Fujizoki Pharmaceutical Co., Ltd.).

Anti-HBs sera. Anti-HBs positive sera were collected from 10 healthy subjects without previous abnormal liver function. Anti-HBs rabbit sera were obtained from 3 rabbits, each of which had been injected with 10 µg HBs subcutaneously 10-14 days earlier. IgG in the pooled rabbit serum was purified by ion exchange chromatography on Q Fast Flow Sepharose (Pharmacia Co., Ltd.). Rabbit immune serum and purified IgG had a passive hemagglutination titer higher than 2⁶. Anti-HBs goat serum was purchased from MBL Co., Ltd. Titration of anti-HBs activity was carried out with the SERODIA-anti HBs assay kit (Fujizoki Pharmaceutical Co., Ltd.).

Polyacrylamide gel electrophoresis (PAGE). This was performed with 12.5% polyacrylamide gel, 1 mm thick for analytical purposes and 1.5 mm thick for preparative purposes, according to the

method of Laemmli (22). Samples containing 5 µg HBs for analytical gels and 100 µg for preparative gels were heated at 60°C for 2 h in electrophoresis sample buffer, 25 mM Tris-HCl (pH 8.3) containing 3% sodium dodecylsulfate (SDS), 5% 2-mercaptoethanol and 10% glycerol. Proteins were visualized after electrophoresis by the silver staining method of Goldman *et al.* (23). Densitograms were obtained with a scanning densitometer (Bio Rad). The molecular weight was determined from the relative mobilities of protein markers (Bio Rad) electrophoresed in parallel.

Reconstitution of HBs polypeptides. Each single band of the HBs polypeptides was cut from the gel, smashed to small pieces in a glass tube containing 1 ml of electrophoresis sample buffer without 2-mercaptoethanol and extracted by vigorous stirring for 16 h at 4°C. Polypeptides extracted from two bands adjusted to the same staining intensity were transferred to a glass tube, mixed for another 16 h in sample buffer without 2-mercaptoethanol, concentrated in a concentrator (Molcut II GC: MILLIPORE) and applied to an analytical SDS-PAGE gel unless otherwise specified.

Western blotting. After electrophoresis, HBs polypeptides were electrotransferred to a sheet of Durapore membrane (MILLIPORE) in 25 mM Tris-Glycine buffer (pH 8.3) containing 30% methanol with constant current of 0.3 ampere at 10°C for 12 h using an electroblotting apparatus (Bio Rad). The membrane was soaked in 20 mM, pH 7.5 Tris HCl buffer-0.15 M NaCl (TBS) containing 0.5% Nonidet P-40 at 4°C for 16 h, washed 3 times with 0.05% Tween 20 in TBS and treated with 3% egg albumin (Difco Co., Ltd.) in TBS for 2 h to block the remaining active sites. HBs polypeptides were immune-stained with anti-HBs sera diluted 20-fold for human and rabbit serum and 1,000-fold for purified rabbit IgG, goat anti-HBs serum and rabbit anti-human serum albumin for 2 h, followed by incubation for 2 h with 1,000-fold diluted horse radish peroxidase-conjugated IgG (Bio Rad) specific for the respective first antibody. TBS containing 0.05% Tween 20 was used to wash the membranes after incubation with antisera, and 3% egg albumin in TBS was used to dilute antisera. Color development of the membrane was carried out by soaking in 0.04% 4-methoxy-1-naphthol (Aldorich Chemical Co., Inc.) in TBS containing

0.01% H_2O_2 , and the reaction was stopped by transferring the membrane to distilled water.

Results

Polypeptide composition of HBs and anti-HBs rabbit serum. Purified HBs was composed of 7 bands at the positions of 24 K, 27 K, 33 K, 36 K, 39 K, 43 K, 67-72 K daltons in which the 24 K and 27 K bands were the major constituents (Fig. 1). Commercial anti-HBs goat serum immune-stained all 7 bands with similar intensity as that obtained by silver staining. On the other hand, pooled anti-HBs rabbit sera obtained from 3 rabbits immune stained only two bands at 60 K and 54 K daltons as shown in Fig. 2. No band was detected in the gel by silver staining method at the position of these molecular weights. Passive hemagglutination titers of goat and rabbit sera were over 2^{10} and 2^6 , respectively. The HBs antigen source and the procedure for immunization were different between the two animals. Commercial goat antiserum might be hyperimmune serum since it was prepared with several booster injections. Although the purity of the HBs antigen preparation was not announced, the anti-

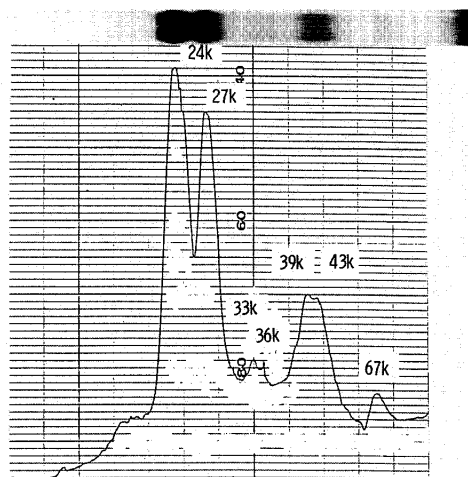


Fig. 1 Silver staining profiles of purified HBs polypeptides.

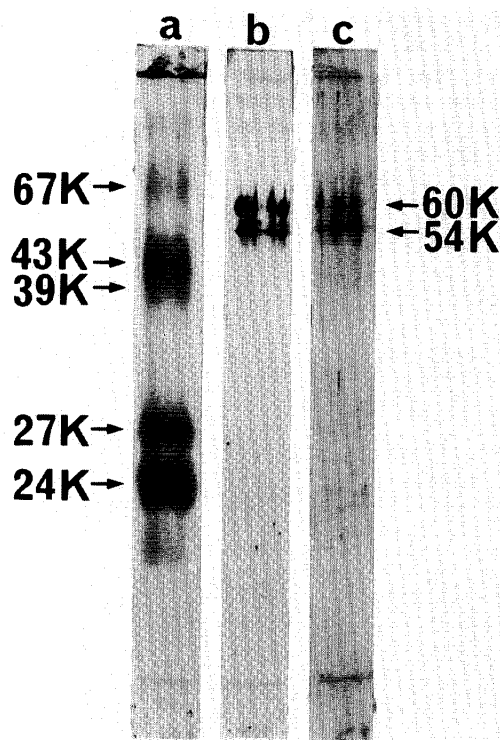


Fig. 2 Immunoblotting profiles of HBs. Immunoblotting using HBs of Fig. 1 as immunoabsorbent was performed with anti-HBs sera of goat (a); rabbit purified IgG (b); and rabbit (c).

serum did not cross-react with human serum in immunoelectrophoresis. The rabbit serum was probably not hyperimmune, since two rabbits out of the 3 were bled 10 days after the first immunization and the other was bled after 14 days with one booster injection at the 10th day. Purified IgG of the rabbit serum did not alter the staining profile.

Anti-HBs human serum. Anti-HBs human sera collected from 10 healthy subjects who were infected with HBV but without any clinical history, were examined to find if they would bind the 60 K and 54 K components of HBs as did the rabbit serum. Six out of the 10 human sera immune-stained 2 bands at 60 K and 54 K daltons as shown in Fig. 3. The remaining four had low passive hemagglutination titers of 2^3 – 2^5 and did not stain any part of the membrane. The six se-

Fig. 3 Immunoblotting profiles of anti-HBs human serum. Ten human serum samples (a-j) were examined. Passive hemagglutination test titers were 2^4-12 for subjects a-f and 2^3-5 for subjects g-j.

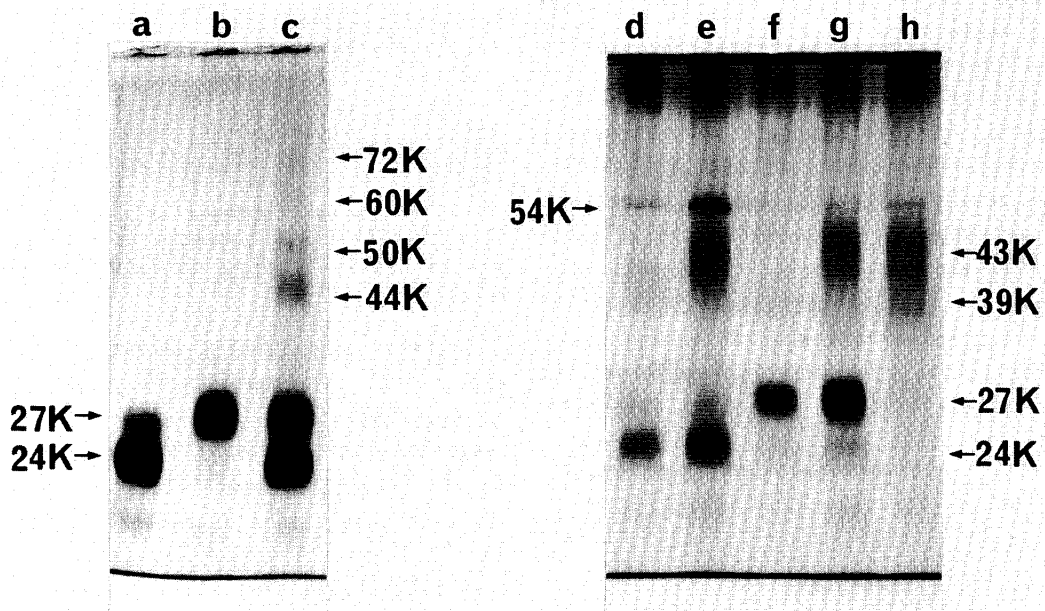
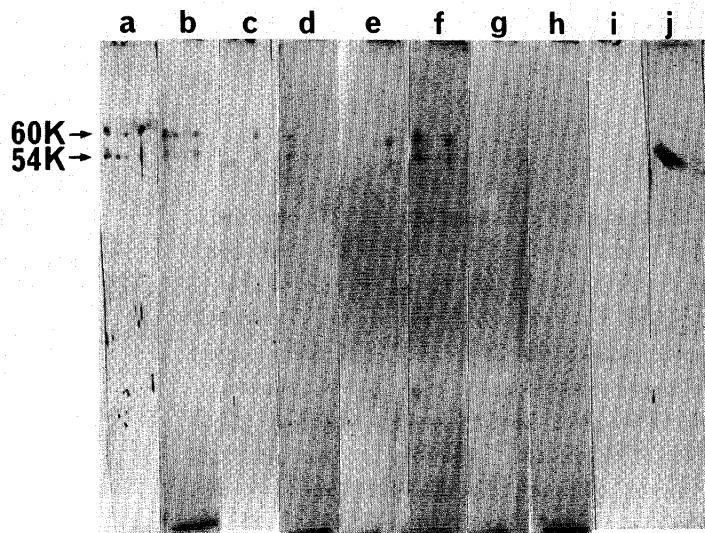


Fig. 4 Silver staining profiles of reconstituted HBs polypeptides. HBs polypeptides extracted from a preparative gel were extracted and the following combinations of the polypeptides were re-electrophoresed: a, 24 K; b, 27 K; c, 24 K+27 K; d, 24 K+39 K; e, 24 K+43 K; f, 27 K+39 K; g, 27 K+43 K; h, 39 K+43 K.

rum samples with positive staining had titers of 2^4-2^{12} .

Reconstitution of HBs polypeptides. Five HBs polypeptides electrophoresed at 24 K, 27 K, 39 K, 43 K, 67-72 K daltons were extracted from the gel and reconstituted by incubation without reducing reagents. The

polypeptides at 33 K and 36 K daltons were not extracted due to low recovery. Only two combinations, 24 K and 27 K, and 24 K and 43 K, resulted in new bands at higher molecular weights. The former gave 44 K, 50 K, 60 K and 72 K bands, and the latter gave only a 54 K band (Fig. 4). Of these 5 new bands,

only 60 K and 54 K bands were immune-stained with pooled rabbit sera. The other combinations of the polypeptides did not give any new bands. The original HBs polypeptides were further denatured by incubation in the electrophoresis sample buffer with 5% 2-mercaptoethanol at 100°C for 5 min and electrophoresed again to check for contamination by polymeric forms. Only the 67-72 K band gave bands at smaller molecular weights of 24 K and 27 K, with less intensity than the 67-72 K band (Fig. 5). These results indicated that the 60 K and 54 K dalton HBs components were composed of 24 K and 27 K polypeptides and 24 K and 43 K polypeptides, respectively, and disclosed an antigenic site recognizable by anti-HBs, which failed to bind respective polypeptides. These results further suggested that the 60 K and 54 K dal-

ton HBs components immune-stained with human and rabbit serum might correspond to these HBs polypeptides in polymeric form. To see whether the 60 K and 54 K polypeptides are directly generated by disrupting the original antigen, HBs was mildly reduced prior to electrophoresis by decreasing concentrations of 2-mercaptoethanol in the sample buffer. With less than 1% of 2-mercaptoethanol, a large amount of HBs was stacked on top of resolving gel, whereas, between 5 and 1%, the separation profile was essentially the same as that in the standard condition with 5% of 2-mercaptoethanol. Only when excess HBs was loaded to the gel in a preparative scale, the 60 K and 54 K bands became faintly visible by the silver staining method.

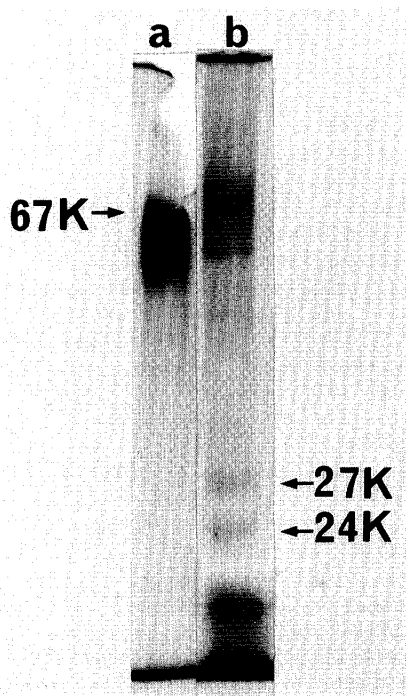


Fig. 5 Silver staining profile of 67-72 K band. A broad band at 67-72 K daltons was extracted from a preparative gel and re-electrophoresed. a, non-treated; b, further denatured with 5% 2-mercaptoethanol and 3% SDS at 100°C for 5 min.

Discussion

The antigen structure of HBs mainly recognized by anti-HBs human and rabbit antibody was investigated by immunoblotting. The results indicated that this antigen structure was the polymeric form of HBs polypeptides. Two polymeric forms, which appeared as 60 K and 54 K bands, might consist of 24 K and 27 K polypeptides and 24 K and 43 K polypeptides, respectively.

The HBs we used was separated into 7 bands at 24 K, 27 K, 33 K, 36 K, 39 K, 43 K and 67-72 K daltons by SDS-PAGE. The 6 bands at smaller molecular weights than the 67-72 K band were probably the same principal HBs polypeptides reported by others (10) because of the similarity in the respective molecular weights and the similar immunoreactivity with commercially purchased anti-HBs antibody. The 67-72 K band might not be a single polypeptide, since it appeared as a broad band and gave 24 K and 27 K bands in addition to the original band after re-electrophoresis. These results suggest

that the 67-72 K band is an aggregation of known HBs polypeptides. Human serum albumin as a contaminant of HBs preparations has been shown to comigrate with the protein band in this region(24-26). However, the 67-72 K band observed in this study should represent, at least in some part, the HBs-specific polypeptides because: 1) HBs we used did not bind to the anti-human serum albumin affinity column(data not shown), 2) 67-72 K band of HBs was not immune-stained with anti-human serum albumin(data not shown), 3) repeated denaturation with 2-mercaptoethanol and SDS did not completely break down the band, and 4) the band was strongly immune-stained with anti-HBs without detectable crossreactivity to human serum albumin. Anti-HBs generated in humans was reported to have immunoreactivity to the group specific antigen *a* of HBs(4). This epitope is known to be present on a certain region of the 6 principal polypeptides(13-15). Thus, anti-HBs human serum was expected to evenly immune-stain the 6 HBs polypeptides as goat serum did. In fact, however, anti-HBs human serum obtained from 10 subjects latently infected with HBV mainly stained only 2 bands at 60 K and 54 K. These 2 bands were hardly detected by silver staining, but were weakly immune-stained with goat serum. A polymeric HBs polypeptide of 49 K daltons generated by mild denaturation followed by column chromatography was reported to produce anti-HBs more potently than the single components of the polypeptides(16). This evidence suggests that the epitope which is mainly recognized by the immune system *in vivo* has a polymeric structure. Our results also suggest a polymeric structure of the epitope, though the molecular weights we determined do not coincide with those reported. Recently, it has been reported that the pre-S region of HBs polypeptides present on 33 K, 36 K, 39 K and 43 K peptides(10) might be a strong antigenic site(19), though the anti-

HBs we used did not react with these polypeptides recognizable by anti pre-S antibody. The anti-HBs we used was from normal subjects latently infected with HBV without a history of acute hepatitis. It must be taken into consideration that anti-HBs from patients with type B acute or chronic hepatitis and subjects who have received HBs vaccine might be different in reactivity to HBs polypeptides.

It must also be taken into consideration that the immunoglobulin class of anti-HBs and the immune state, *i.e.*, whether heavily or weakly immunized, might affect the generation of anti-HBs with different reactivity to HBs polypeptides.

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